SUPPORTING INFORMATION

A "Smart" ^{129}Xe NMR Biosensor for pH-Dependent Cell Labeling

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CONTENTS

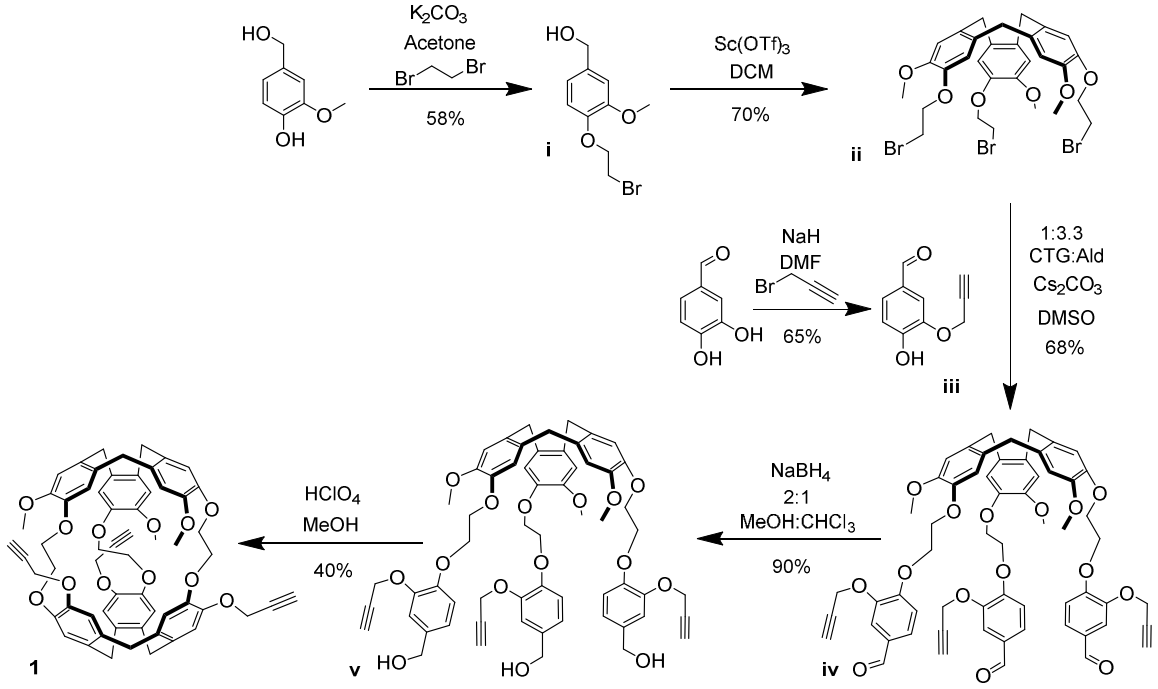
General Information

Instrumentation and Methods. ^{1}H NMR (500 MHz) data were obtained in deuterated chloroform ($CDCl_3$) or dimethyl sulfoxide (DMSO-*d6*) using a Bruker DMX 500 NMR spectrometer. Column chromatography was performed using silica gel (60 Å pore size, 40-75 μm particle size) from Sorbent Technologies. Thin layer chromatography (TLC) was performed using silica gel plates (60 Å pore size, Silicycle) with UV light at 254 nm as the detection method. MALDI-MS data were collected using a Bruker Ultraflex III TOF/TOF mass spectrometer. All HPLC purifications were performed on a Varian Prostar 210 system equipped with a quaternary pump and diode array detector. All air- and moisture-sensitive reactions were performed under inert atmosphere in glassware flamed under vacuum, using anhydrous dry solvents. Standard workup procedures involved multiple (~3) extractions with the indicated organic solvent, followed by washing of the combined organic extracts with water or brine, drying over Na_2SO_4 and removal of solvents *in vacuo*. All yields reported were determined after purification by column chromatography or reverse phase HPLC. All data were collected using instruments in the Chemistry Department at the University of Pennsylvania.

Materials. Organic reagents and solvents were used as purchased from the following commercial sources: Sigma-Aldrich: N,N-diisopropylethylamine (DIPEA); dimethyl sulfoxide (DMSO, anhydrous, 99.9%); Sigmacote®. Fisher: acetone (HPLC grade); chloroform (CH_2Cl_3, HPLC grade); dichloromethane (CH_2Cl_2, HPLC grade); ethyl acetate (EtOAc, HPLC grade); hexanes (HPLC grade); hydrochloric acid; methyl alcohol (MeOH, HPLC grade), perchloric acid (60%); Pluronic L-81; potassium carbonate (anhydrous); sea sand (washed); sodium chloride (NaCl); sodium hydroxide (NaOH); sodium sulfate (anhydrous). Novabiochem (currently EMD Millipore; Billerica, MA, USA): 6-azidohexanoic acid; 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). MarCor: deionized (DI) water filtered (18 MΩ). Acros Organics: 3-bromopropionic acid (97%); cesium carbonate (Cs_2CO_3, 99.5%); chloroform-*d* ($CDCl_3$); 1,2-dibromoethane; 3,4-dihydroxybenzaldehyde (97%); N,N-dimethylformamide (DMF, 99.8%, anhydrous, acrosseal); dimethylsulfoxide-*d6*, 4-hydroxy-3-

methoxybenzyl alcohol (99%); propargyl bromide (80% solution in toluene); scandium(III) trifluoromethanesulfonate ($Sc(OTf)_3$, 95%); sodium borohydride ($NaHB_4$, powder, 98%); sodium hydride (NaH, 60% dispersion in mineral oil); tetrahydrofuran (THF, extra dry, over molecular sieves); triisopropylsilane (TIS). MG Industries (Linde Group, NJ): xenon gas (scientific grade).

SYNTHETIC PROCEDURES



Scheme S1: 6-step, non-linear synthesis of tripropargyl cryptophane (**1**). Yield = 9.9% for the five linear steps.

Cryptophane Synthesis. Tripropargyl cryptophane was achieved in a 6-step synthesis with a 6.4% overall yield from two commercially available compounds, 3,4-dihydroxybenzaldehyde and vanillyl alcohol shown in Scheme **S1**.

4-((2-bromoethoxy)-3-methoxyphenol) methanol (**i**): In a dry two-necked flask with a nitrogen inlet, vanillyl alcohol (10.0 g, 64.9 mmol, 1 eq) was combined with potassium carbonate (44.8 g, 324 mmol, 5 eq) in acetone (100 mL) and stirred at rt for 30 min. 1,2-dibromoethane (56.3 mL, 650 mmol, 10 eq) was then added to the reaction via syringe. The reaction flask was then transferred to a pre-heated oil bath at 57 °C and refluxed overnight. The organic components were isolated with EtOAc on aqueous workup and

the final product was purified by column chromatography (1:1 to 7:3 hexanes:EtOAc, gradient method; TLC 1:1 hexanes:EtOAc R_f(**i**) = 0.43) to yield 10.2 g (39.1 mmol, 60% yield). The spectroscopic data match those reported in the literature.[1]

2,7,12-Tris-(2-bromoethoxy)-3,8,13-trimethoxy-10,15-dihydro-2H-tribenzo[a,d,g]cyclononene (**ii**) was prepared in 70% yield (5.19 g, 7.12 mmol) as previously described.[1]

3-propargyloxy-4-hydroxybenzaldehyde (**iii**) was prepared in 65% yield (5.72 g, 32.5 mmol) following our published protocols,[2] with the only variation being the use of DMF as the solvent for reaction at 0 °C.

2,7,12-Tris-[2-[4-formyl-2-propargyloxyphenoxy]ethoxy]-3,8,13-trimethoxy-10,15-dihydro-2H-tribenzo[a,d,g]cyclononene (**iv**): prepared as previously described with a 68% yield (1.08 g, 1.06 mmol).[1]

2,7,12-Tris-[2-[4-(hydroxymethyl)-2-propargyloxyphenoxy]ethoxy]-3,8,13-trimethoxy-10,15-dihydro-2H-tribenzo[a,d,g]cyclononene (**v**) was prepared by substituting chloroform for THF to increase solubility.[1] Reaction progress was monitored by TLC (5% MeOH in CH_2Cl_2). Additional equivalents of $NaHB_4$ were added as necessary, until quantitative conversion was observed. No further purification was necessary after aqueous work-up. In this manner, >90% yield (0.971 g, 0.951 mmol) was achieved.

Tripropargyl Cryptophane (**1**): Methanol (200 mL) was added to a 1 L reaction flask containing compound **v** (50.0 mg, 0.0490 mmol, 1 eq). The reaction was put to stirring in a salted ice bath (-10 °C) and fitted with an addition funnel. Perchloric acid (60%) (150 mL) was added drop-wise over several hours. Once addition was complete, the reaction was allowed to slowly warm to rt and stir overnight. Afterwards, the reaction was again placed on a salted ice bath and more perchloric acid (50 mL) was added. The reaction was monitored by TLC (5% acetone in CH_2Cl_2) and subsequent 25 mL perchloric acid additions were performed as necessary to drive the reaction to completion. The reaction was quenched with the addition of dH_2O and brine (200 mL each) and then extracted 3x with CH_2Cl_2 (100 mL). The combined organics were then washed with dH_2O, sat. sodium bicarbonate, and brine (100 mL), dried over sodium sulfate and concentrated *in vacuo*. The crude material was purified by silica gel flash column chromatography (5% acetone in CH_2Cl_2, *v/v*) to yield 20.0 mg (0.0207 mmol, 40% yield) of **1** as a

white powder. TLC (silica gel, 5% acetone in CH_2Cl_2, *v/v*); R_f(**1**)= 0.47. The spectroscopic data match those reported in the literature.[1,3]

Peptide Synthesis and Purification. The EALA-repeat peptide, sequence: WEAALAEALAEALAEHLAEALAEALEALAA, was synthesized (100 micromole scale) by solid-phase peptide synthesis, using Fmoc chemistry, on a Liberty 1 Automated Microwave Peptide Synthesizer located in the UPenn Biological Chemistry Resource Center. Piperidine (20%) in DMF was used as the deprotection agent, 0.5 M HBTU in DMF was used as the activator, and 2 M DIPEA in N-methyl-2-pyrrolidone (NMP) was used as the activator base. Five molar equivalents of the amino acid were used for each coupling on Rink Amide MBHA resin (0.59 mmol/g substitution, Novabiochem). Residue Ala_{30} used method 1 and His_{16} used method 3 and all other residues used method 2. Method 1: Initial 30 s microwave deprotection (35 W, 75 °C), followed by 3-min microwave deprotection (35 W, 75 °C). Two consecutive coupling cycles were used. Both couplings were for 5 min under microwave power (25 W, 75 °C). The instrument was then paused and taken off-line while the unreacted resin was acetylated with a mixture of 2 mL acetic anhydride, 1.2 mL of *N*-methylmorpholine (NMM), and 16.8 mL of DMF. Method 2: Initial 30-s microwave deprotection (35 W, 75 °C), followed by a 3-min microwave deprotection (35 W, 75 °C). Two consecutive coupling cycles were used. Both couplings were for 5 min under microwave power (25 W, 75 °C). Method 3: Initial 30-s microwave deprotection (35 W, 75 °C), followed by a 3-min microwave deprotection (35 W, 75 °C). Two consecutive coupling cycles were used. Both couplings were for 2 min at rt followed by 3 min under microwave power (25 W, 50 °C). The peptide then underwent a final deprotection (initial 30 s microwave deprotection (35 W, 75 °C), followed by a 3 min microwave deprotection (35 W, 75 °C) leaving the peptide on resin with a free N-terminus.

Azido-EALA peptide (**2**): The EALA peptide on resin in a sigmacoted vessel (0.1 mmol, 1 eq) was incubated in an excess (~10 mL) of DMF with magnetic stirring for 30 min to allow for resin swelling. The DMF was subsequently removed by vacuum suction. The 6-azidohexanoic acid (78.6 µL, 0.5 mmol, 5 eq) was combined with DMF (6 mL), HBTU (190 mgs, 0.5 mmol, 5 eq) and DIPEA (175 µL, 1.0 mmol, 10 eq) and added to the resin under magnetic stirring for 1 h. The reagent mixture was drained

with vacuum suction and the resin was iteratively washed with MeOH, DCM, and DMF and then drained with vacuum. The resin was subjected to a second round of coupling with the same reagent mixture. After rinsing extensively, the resin was dried over DCM for 30 min on vacuum. The azido peptide was then dosed with a cleavage cocktail of 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS), and 2.5% dH_2O and stirred for 2.25 h. The vessel was drained and the resulting solution was collected and reduced to approximately 1 mL on a rotary evaporator. The peptide was then precipitated from solution with cold diethyl ether and the resulting solid was isolated by centrifugation. The peptide was resuspended in HPLC solvents (1:1 acetonitrile:dH_2O with 0.1% TFA). The azido-EALA peptide was purified using reverse-phase HPLC and monitored at 215 and 280 nm using a Zorbax RxC8 semi-preparative column (9.4×250 mm, 5μm beads). The elution gradient was composed of two solvents: 0.1% aqueous TFA (solvent A) and a 0.1% solution of TFA in CH_3CN (solvent B). The purification method went from 65% A to 40% A over 5 min, then from 40% A to 25% A over 5 min, and then from 25% A to 0% A over 35 min at a flow rate of 4 mL/min and with a 1 mL injection volume. The peptide eluted at 21.04 min, Figure **S1**. MALDI MS m/z calculated for N_3-EALA $C_{142}H_{225}N_{37}O_{45}$ ($M+H^+$) 3169.65; found 3169.51, Figure **S2**.

Solubilizing Linker Synthesis. 3-Azidopropionic acid was prepared from β-propiolactone by literature procedure and matched the reported ^1H NMR spectrum.[4]

3-azidopropionic acid (**4**). Briefly, sodium azide (4.5 g, 0.69 mmol, 1 eq) was dissolved in MilliQ water. β-propiolactone (4.4 mL, 0.069 mmol, 1 eq) was added dropwise and the reaction was allowed to stir at rt for 6 h. The reaction was neutralized with 1 M HCl and then extracted 3 times with diethyl ether. The organic layer was then dried over sodium sulfate and filtered through cotton. A clear oil in a 15% yield was recovered, requiring no further purification.

WEC Biosensor Synthesis. The copper(I)-catalyzed [3+2] azide-alkyne cycloaddition (CuAAC) between N_3-EALA peptide and cryptophane and the subsequent cycloaddition reaction between the cryptophane and 3-azidopropionic acid yielded the WEC biosensor**.**

EALA-cryptophane (**3**). To conjugate the azido-EALA peptide to the tripropargyl cryptophane (**1**) CuAAC was utilized with modified conditions of those previously employed.[2,5-7] Firstly, 4 mg (1.0 eq) of

1 and 13 mg of **2** (1 eq) were dissolved in 1 mL of dry methyl sulfoxide (DMSO) in a conical reaction vessel along with 1 cm of 18 g copper wire. The reaction mixture was put to stirring and degassed. In a separate vial, 10 mg (5 eq) of tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methylamine (TBTA) copper ligand was dissolved in 60 μL of DMSO. Separate solutions of 0.6 M copper (II) sulfate ($CuSO_4$) and 4 M sodium ascorbate (NaAsc) in water were also prepared. To the TBTA solution, 6 μL of $CuSO_4$ (1 eq), 0.5 μL of 2,6-lutidine (1 eq), and 9 μL (+)-sodium-L-ascorbate (10 eq) were added sequentially, vortexing between additions. After addition of NaAsc, the mixture turned clear indicative of Cu(I) formation. The entire reagent mixture was then added to the reaction vessel. The reaction vessel was again degassed and then covered with foil and allowed to stir overnight under nitrogen at rt. A small aliquot of reaction was removed (10 μL) and diluted in an HPLC solvent mixture, 50:50 mixture of acetonitrile and water with 0.1% trifluoroacetic acid. To verify product formation, analytical reverse-phase HPLC was performed using a Zorbax RxC8 analytical column (4.6×150 mm, 5 μm beads) and monitored at 215 and 280 nm. The elution gradient was composed of two solvents: 0.1% aqueous TFA (solvent A) and a 0.1% solution of TFA in CH_3CN (solvent B). The purification method went from 65% A to 40% A over 15 min, then from 40% A to 18% A over 25 min, and then from 18% A to 0% A over 3 min at a flow rate of 1 mL/min with a 1 mL injection volume. The EALA-cryptophane eluted at 36.50 min, Figure **S3**. MALDI MS m/z calculated for EALA-cryptophane $C_{202}H_{279}N_{37}O_{57}$ ($M+H^+$) 4136.01; found 4136.84, Figure **S4**.

Water-soluble EALA-cryptophane (WEC) (**5**). The crude reaction (**3**) was then dosed with 4.6 μL 3-azidopropionic acid (**4**) (10 eq), and the same reagent mixture as in the previous reaction and allowed to stir overnight while covered, to yield the water-soluble EALA-cryptophane (WEC) biosensor. The reaction was diluted 10-fold in 50:50 ACN:H_2O and purified. Purification was achieved through reverse-phase HPLC employing the semi-preparative version of the previous method by using a Zorbax RxC8 semi-preparative column (9.4×250 mm, 5 μm beads) with 4 mL/min flow rate. The pH sensor eluted at 33.87 min, Figure **S5**. MALDI MS m/z calculated for WEC $C_{208}H_{289}N_{43}O_{61}$ ($M+H^+$) 4366.08; found 4366.36, Figure **S6**. The pure fractions were then collected and diluted with 0.1 % ammonium hydroxide

to 10% acetonitrile. Amicon-ultra-4mL 3K NMWL Centricon tubes were used to concentrate and buffer exchange the final product **5** into 10 mM sodium phosphate buffer at pH 7.5.

CHARACTERIZATION METHODS

Electronic Circular Dichroism (ECD) Spectroscopy. All spectroscopy experiments were performed on the Aviv 410 CD spectrometer. Data were collected at 25 °C from 260-190 nm, with a 30 s averaging time, 1 nm wavelength step, 1 s averaging time, and 1 nm bandwidth. The resulting ellipticity measurements (Θ_D) were converted to molar residue ellipticity (Θ) values using: $\theta = \theta_D/Cln_R$ where C is concentration (M), l is path length (cm), and n_R is the number of residues. The samples were prepared by concentrating the purified WEC to 30 μM in 10 mM sodium phosphate at pH 7.5 and aliquoting the stock into five Eppendorf tubes and adjusting the pH to 5.5, 6.0, 6.5, 7.0, and 7.5 with a few microliters of 1 M HCl. The concentration was confirmed by measuring the absorbance at 280 nm, ε_{280} = 17,700 $M^{-1}\ cm^{-1}$ and using an Agilent 89090A UV-visible spectrophotometer.

Data Analysis. The molar ellipticity was calculated from the observed ellipticity (mdeg) and has the units of deg cm^2 dmol^{-1}. The molar ellipticity is given by equation **S1** where *C* is the concentration of the peptide or biosensor, ℓ is the path length of the cuvette-0.1 cm, and n_r is the number of residues-31.[8]

$$[\theta] = \frac{\theta_\lambda}{(C * \ell * n_r * 10)} \quad \text{(S1)}$$

The helical content of the peptide and biosensor was determined from circular dichroism studies at 30 μM concentrations and in accordance with literature precedent.[9] Helicity was calculated using the formulas **S2** and **S3** where n_r is the number of amide bonds in the peptide, in this case 31. Racemic cryptophane was employed for these experiments, and thus did not contribute to the measured CD signal. Data are shown in Tables **S1** and **S2** and graphically in Figure **S7**.

$$\%helicity = 100 * \frac{[\theta]_{222}}{max[\theta]_{222}} \quad \text{(S2)}$$

$$^{max}[\theta]_{222} = -40000 * [1 - \frac{2.5}{n_r}] \quad \text{(S3)}$$

pH Reversibility. Repeated trials with the azido-peptide alone and with WEC showed that the peptide conformational change was reversible. Whether the sample was first prepared at pH 5.5 or at pH 7.5 and titrated to higher or lower pH in this range, the ECD signal confirmed the conformation aligned with what is shown in Figure **1**. One example of this reversibility is shown in Figure **S8**. In this experiment the peptide sample at pH 7.5 shown in Figure **1a** was titrated to pH 5.5 and the CD signal was measured. The same sample was then titrated back to pH 7.5 (labeled pH 7.5R) and the CD signal was measured and found to align with the original spectrum at pH 7.5.

Tryptophan Fluorescence Studies. All fluorescence studies were carried out on Photon Technology International (PTI) QuantaMaster™ 40 fluorescence spectrometer (Birmingham, NJ, USA). The 30 μM samples at pH 5.5, 6.0, 6.5, 7.0, and 7.5 were removed from the CD cuvettes and placed in the fluorometer. Fluorescence spectra were collected at 25 °C in quartz cuvettes with a 1-cm path length. The samples were excited at 280 nm and emission data were collected from 300-400 nm. For all spectra the slit widths were 5 nm, scan rate was 60 nm/min, averaging time was 1 s, and the data interval was 1 nm.

Hyperpolarized ^{129}Xe NMR Spectroscopy. HP ^{129}Xe was generated using spin-exchange optical pumping (SEOP) method with a home-built version of the previously commercially available Nycomed-Amersham (now GE) model IGI.Xe.2000 ^{129}Xe hyperpolarizer. A gas mixture of 89% helium, 10% nitrogen, and 1% natural abundance xenon (Linde Group, NJ) was used as the hyperpolarizer input. 795 nm circularly polarized diode laser was used for optical pumping of Rb vapor. ^{129}Xe was hyperpolarized to 10–15% after being cryogenically separated, accumulated, thawed, and collected in controlled atmosphere valve NMR tubes (New Era). After hp Xe collection, NMR tubes were shaken vigorously to mix cryptophane solutions with hp Xe. All ^{129}Xe NMR measurements were carried out on a Bruker BioDRX 500 MHz NMR spectrometer (138.12 MHz frequency for ^{129}Xe), using a 10-mm BBO NMR probe. Sample temperature was controlled by VT unit on NMR spectrometer to 300 ± 1 K. Eburp2 shaped pulse was used to selectively excite Xe@WEC biosensor peak. Spectra were averaged over 16 scans. A

delay of 0.5 s was given between scans to allow for xenon exchange. All acquired NMR spectra were processed with 60 Hz Lorentz broadening. Chemical shifts were referenced to free xenon gas of 0 atm at 0 ppm, shown in Figure **3**.

Hyper-CEST Frequency Scan Spectroscopy. Prior to applying saturation pulse, gas mixture including HP Xe was bubbled into a 10-mm NMR tube containing 2.5 mL sample solution by a home-built continuous-flow gas delivery setup. For each data point in the Hyper-CEST spectrum, the gas mixture was bubbled for 20 s, followed by a 3-s delay to allow bubbles to collapse. All Hyper-CEST experiments were carried out using a Bruker 500 MHz NMR spectrometer, with 10-mm PABBO probe. A 90° hard pulse of this probe has pulse length of 22 μs.

Hyperpolarized ^{129}Xe Chemical Exchange Saturation Transfer Depolarization Curve. Saturation frequencies of Dsnob shaped pulse were positioned at (192.4 - 128.2) = 64.2 ppm and (192.4 + 128.2) = 320.6 ppm, for "on" and "off" resonance, respectively. In each experiment, pulse sequences of the following parameters were used: Pulse length t_{pulse} = 3.748 ms, field strength $B_{1,max}$ = 77 μT, delay between pulse = 20 μs, maximum number of saturation cycles = 6000. Data are shown in Figure **5**. Sample temperature was controlled by VT unit on NMR spectrometer to 310 ± 1 K. Xenon was introduced by continuous flow.

Cell Culture. Human cervical carcinoma (HeLa) cells in T-25 cell culture flasks in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin were grown to 80% confluency. The medium was then removed and the cells were washed 3x with Dulbecco's Phosphate Buffered Saline (DPBS). Cells were suspended with 0.25% trypsin incubation for 5 min. The trypsin was quenched with a 10-fold excess of DPBS. Cell suspension (10 μL) was removed and combined with 10 μL of Trypan Blue. Cells were counted with a hemocytometer after 5-min incubation. 1 x 10^7 cells/mL were used in all experiments. The cell suspension was then centrifuged for 7 min at 2 krpm and the cell pellet was isolated. Cells were resuspended in 10 mM sodium phosphate buffer with

0.1% Pluronic L-81[10] at either pH 5.5 or pH 7.5 and also 5-10 μM WEC. The cell/biosensor solution was gently vortexed to mix. After < 1-h incubation, the cell suspension was transferred to an NMR tube and degassed.

FIGURES:

Table S1: α-helical content of the EALA peptide at various pH values in 10 mM sodium phosphate buffer

pH	$-[\theta]_{222}$ (deg*cm^2*dmol^{-1})	Helicity (%)
5.5	24, 579	67
6.0	22,414	61
6.5	19,415	53
7.0	11,670	32
7.5	9,142	25

Table S2: α-helical content of the WEC biosensor at various pH values in 10 mM sodium phosphate buffer

pH	$-[\theta]_{222}$ (deg*cm^2*dmol^{-1})	Helicity (%)
5.5	22,530	61
6.0	18,825	51
6.5	17,081	46
7.0	14,854	40
7.5	13,390	36

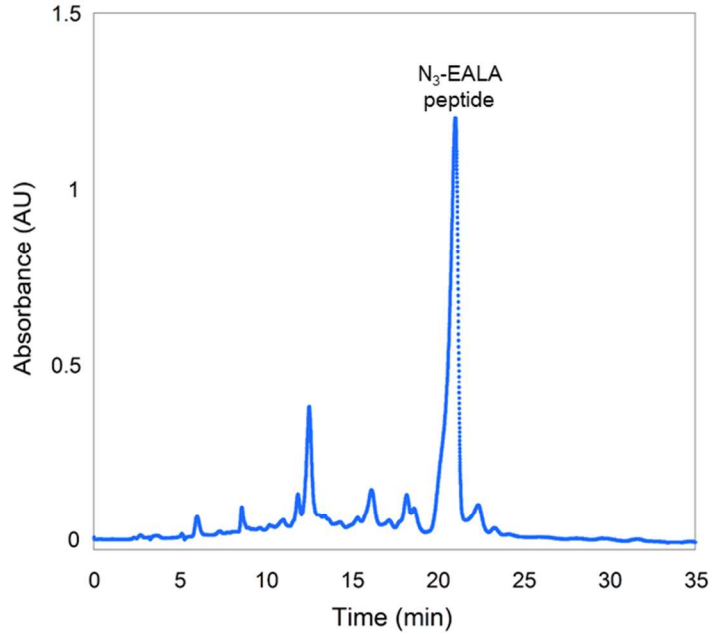


Figure S1: HPLC chromatogram of N_3-EALA (**2**). UV absorbance monitored at 280 nm. Peak assignment based on MALDI-MS.

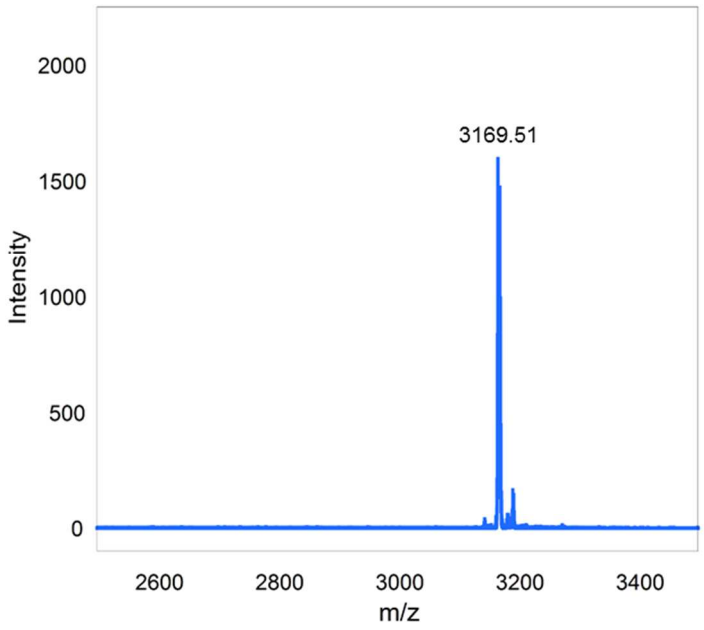


Figure S2: MALDI-MS spectrum of N_3-EALA peptide. Expected mass [$M+H^+$] 3169.65; found 3169.51.

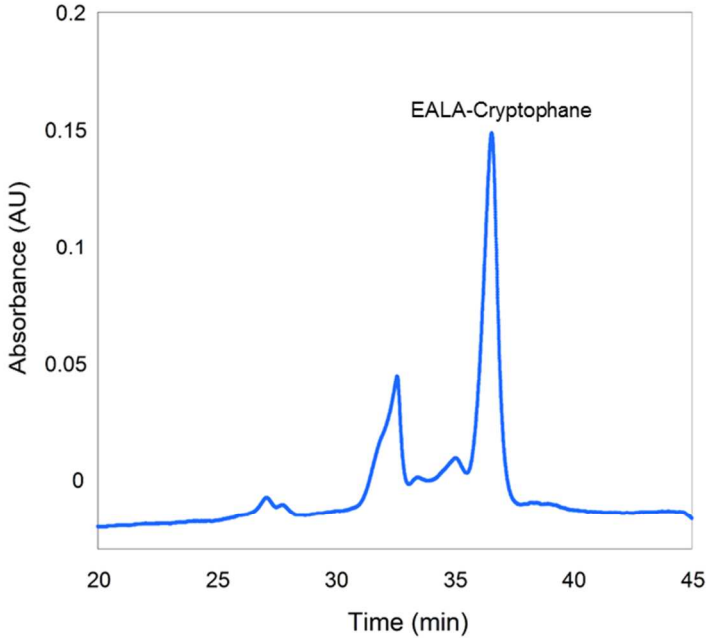


Figure S3: HPLC chromatogram of EALA-cryptophane (**3**). UV absorbance monitored at 280 nm. Peak assignment based on MALDI-MS.

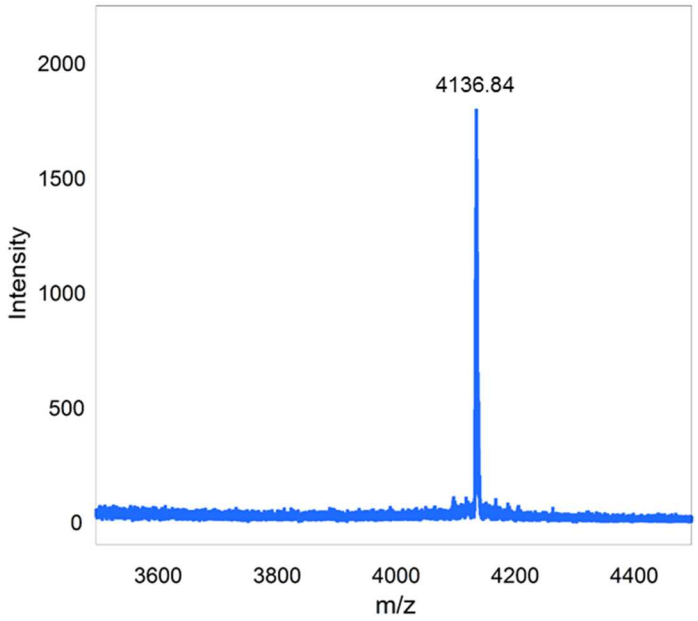


Figure S4: MALDI-MS spectrum of EALA-cryptophane (**3**). Expected mass [M+H$^+$] 4136.01; found 4136.84.

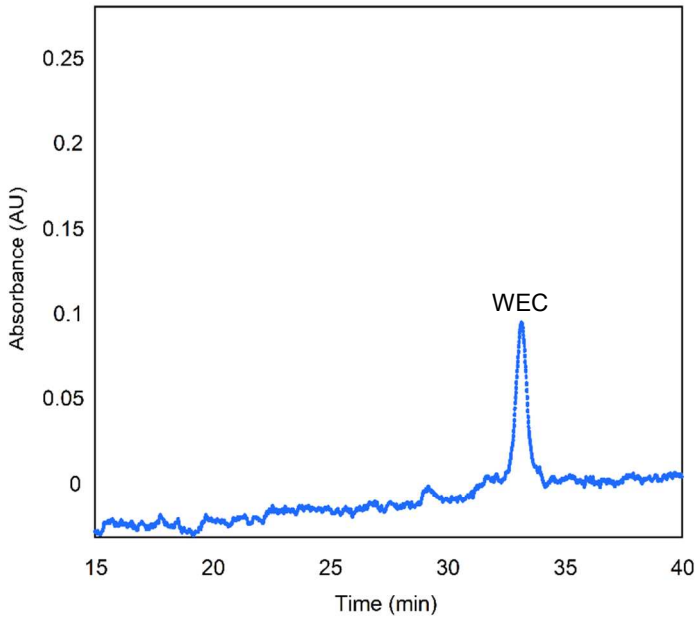


Figure S5: HPLC chromatogram of WEC (**5**). UV absorbance monitored at 280 nm. Peak assignment based on MALDI-MS.

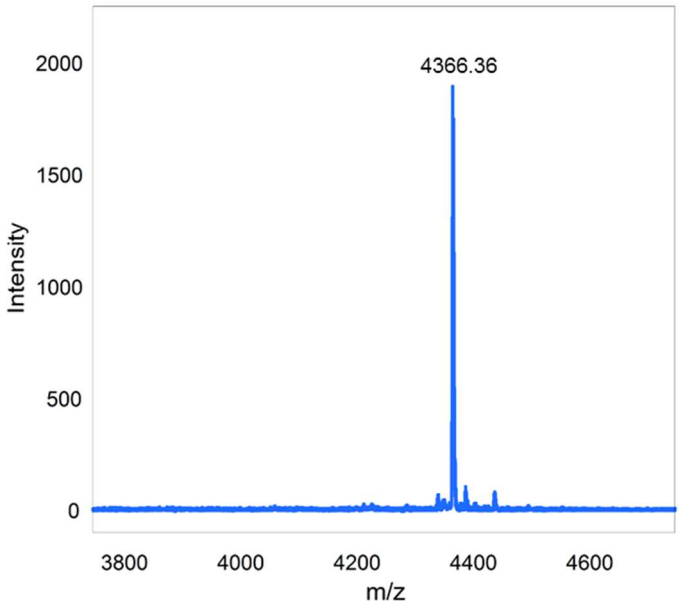


Figure S6: MALDI-MS spectrum of WEC (**5**). Expected mass [M+H$^+$] 4366.08; found 4366.36.

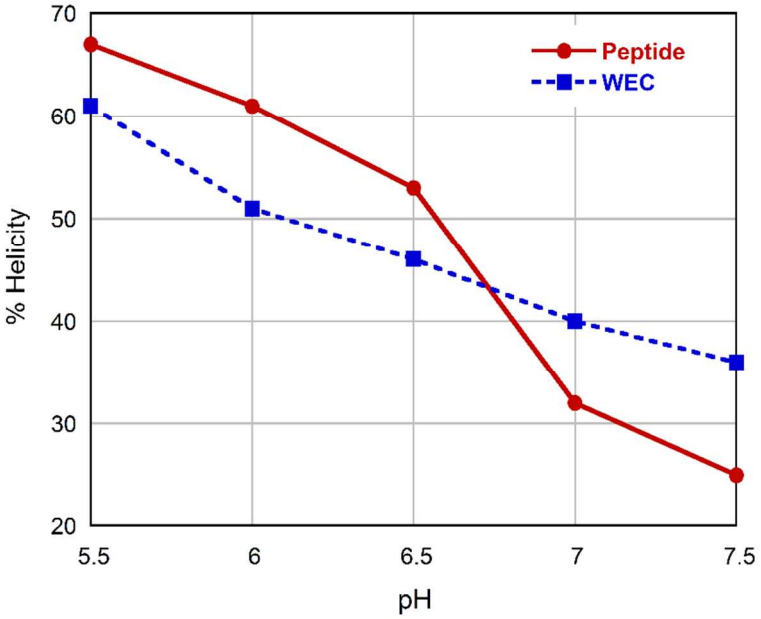


Figure S7: Change in helicity as a function of pH of the azido-peptide (red) and WEC (blue).

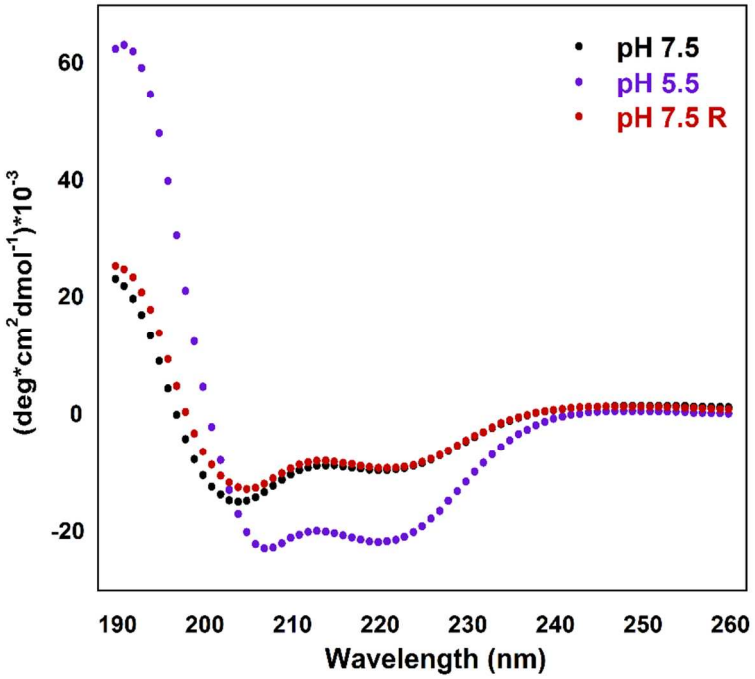


Figure S8: pH reversibility experiment shows the peptide can transition from disordered (pH 7.5, black) to alpha-helical (pH 5.5, blue), back to disordered structure (pH 7.5, red).

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